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REVIEW

The formation of skeletal muscle: from somite to limb

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Abstract

During embryogenesis, skeletal muscle forms in the vertebrate limb from progenitor cells originating in the somites. These cells delaminate from the hypaxial edge of the dorsal part of the somite, the dermomyotome, and migrate into the limb bud, where they proliferate, express myogenic determination factors and subsequently differentiate into skeletal muscle. A number of regulatory factors involved in these different steps have been identified. These include Pax3 with its target c-met, Lbx1 and Mox2 as well as the myogenic determination factors Myf5 and MyoD and factors required for differentiation such as Myogenin, Mrf4 and Mef2 isoforms. Mutants for genes such as *Lbx1* and *Mox2*, expressed uniformly in limb muscle progenitors, reveal unexpected differences between fore and hind limb muscles, also indicated by the differential expression of *Tbx* genes. As development proceeds, a secondary wave of myogenesis takes place, and, postnatally, satellite cells become located under the basal lamina of adult muscle fibres. Satellite cells are thought to be the progenitor cells for adult muscle regeneration, during which similar genes to those which regulate myogenesis in the embryo also play a role. In particular, *Pax3* as well as its orthologue *Pax7* are important. The origin of secondary/fetal myoblasts and of adult satellite cells is unclear, as is the relation of the latter to so-called SP or stem cell populations, or indeed to potential mesangioblast progenitors, present in blood vessels. The oligoclonal origin of postnatal muscles points to a small number of founder cells, whether or not these have additional origins to the progenitor cells of the somite which form the first skeletal muscles, as discussed here for the embryonic limb.

Key words muscle progenitor cells; muscle regeneration; myogenesis; myogenic factors; Pax genes; regulatory genes; vertebrate limb.

Introduction

In vertebrates such as birds and mammals, skeletal muscle forms in the embryo from paraxial mesoderm, which segments into somites on either side of the neural tube and notochord (see Christ & Ordahl, 1995). The ventral part of the somite, the sclerotome, will contribute the cartilage and bone of the vertebral column and ribs, whereas the dorsal part of the somite, the dermomyotome, as its name implies, gives rise to the overlying derm of the back and to the skeletal muscles of the body and limbs. Some of the muscles in the head are derived from anterior, unsegmented, paraxial mesoderm and from prechordal mesoderm.

Most of our detailed understanding of the embryology of myogenesis is based on the manipulation of chick embryos and chick/quail chimaeras; the general description appears to apply also to mammals. In mammals, and particularly in the mouse, where gene manipulation makes it possible to test function, the molecular regulation of myogenesis has been partially established (see Buckingham, 2001). It is clear that the MyoD family of basic helix–loop–helix factors plays a critical role; in double *Myf5*/*MyoD* mutants no skeletal muscle forms because the precursor myoblast population is absent (Rudnicki et al. 1993). In the absence of these factors, cells in the somite, which would normally become myoblasts, do not locate correctly to sites of myogenesis

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and adopt other cell fates (Tajbakhsh et al. 1996), thus demonstrating the role of MyoD and Myf5 as myogenic determination factors. Other families of transcription factors are also implicated in the genetic hierarchy that leads to the formation of skeletal muscle. Pax3, for example, acts, with Myf5, upstream of *MyoD* (Tajbakhsh et al. 1997).

Myoblast cell differentiation into muscle fibres also depends on the MyoD family, MyoD, Mrf4 and, particularly, Myogenin. Other transcription factors also play an important role in this context, notably the Mef2 family, characterized by the presence of an MADS–box motif (see Black & Olson, 1998), and six homeo-domain family members (Spitz et al. 1998; see Relaix & Buckingham, 1999).

In this paper our understanding of how skeletal muscle forms in the limbs of the embryo will be presented and later stages of muscle formation, maturation and regeneration discussed.

Muscle formation in the embryonic limb

Skeletal muscle in the limb is formed by cells derived from somites present at the level of the limb buds. The somite can be divided into epaxial and hypaxial parts according to an anatomical division of the body and its musculature, clearly perceptible in fish for example. The epaxial dermomyotome, adjacent to the neural tube and notochord, gives rise to the deep back muscles whereas the rest of the musculature of the body and the limbs derives from the hypaxial extremity of the dermomyotome. The first muscle mass to form, under the dermomyotome, is the myotome, which has an epaxial and a hypaxial component, subsequently integrated into the trunk musculature (Fig. 1) (see Tajbakhsh & Buckingham, 2000). Opposite the limb buds, muscle progenitor cells delaminate from the epithelium of the hypaxial dermomyotome and migrate into the limb field, to the positions where the dorsal and ventral muscle masses will form initially. It is the mesenchymal cells of the limb which are thought to provide the positional cues for the muscle progenitor cells coming from the somite (see Christ & Ordahl, 1995).

Delamination and migration of muscle progenitor cells

Both delamination and migration depend on the presence of c-met, a tyrosine kinase receptor which interacts with its ligand HGF, also called scatter factor, produced by non-somitic mesodermal cells which thus delineate the migratory route (Dietrich et al. 1999). In mutant mouse embryos which lack functional c-met (Bladt et al. 1995) or HGF (Schmidt et al. 1995), skeletal muscle is absent from the limbs. Transcription of the *c-met* gene depends on Pax3, a transcription factor characterized by the presence of homeo- and paired domain motifs (Epstein et al. 1996). *Pax3* mutant mice also have no limb muscles and cells do not delaminate from the hypaxial dermomyotome (see Tajbakhsh et al. 1997); this can be followed at the cellular level with an nlacZ reporter sequence introduced into an allele of *Pax3* (Fig. 2).

It is unclear how Pax3 functions in the embryo, since on its own it is a very poor transcriptional activator and can even repress transcription (see Magnaghi et al. 1998). However, when a sequence encoding a constitutionally active form of Pax3, the Pax3–FKHR fusion, which results from a chromosomal translocation and can give rise to rhabdomyosarcomas in humans (e.g.

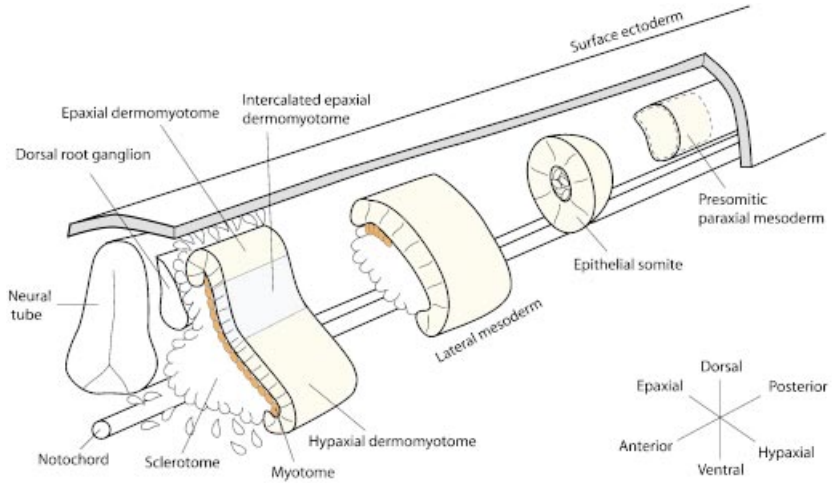

Fig. 1 Schematic representation of somitogenesis.

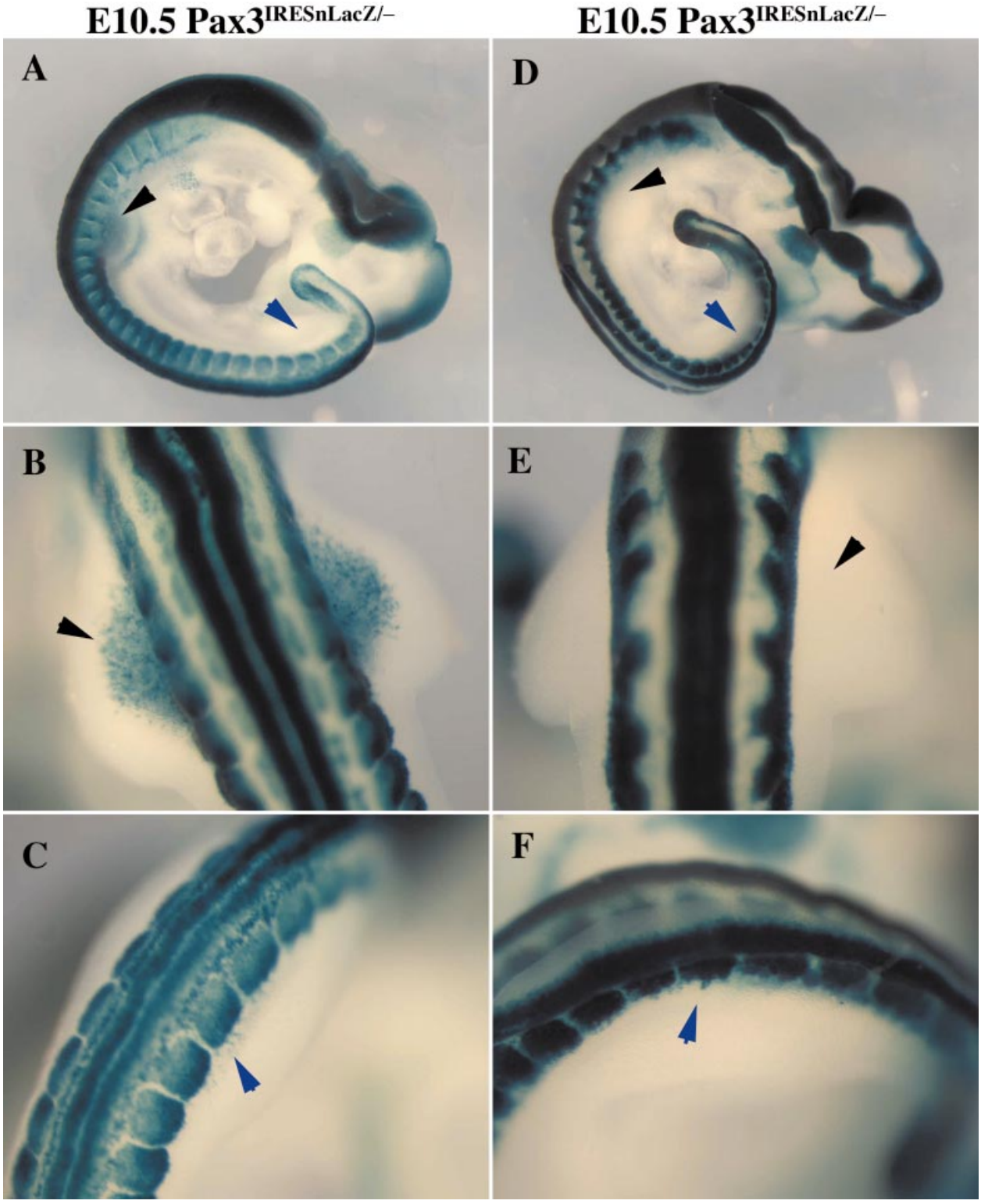

Fig. 2 Mouse embryos at embryonic day (E) E10.5 with one (A–C) or two (D–F) alleles of *Pax3* targeted with an nlacZ reporter sequence. In heterozygote embryos (A–C), β-galactosidase (Pax3)-positive cells can be seen in the forelimb bud (A and B, black arrow). At the hindlimb bud level (A and C), at this stage, β-galactosidase-positive (Pax3) cells delaminating from the somites and beginning to migrate are seen (blue arrow). In homozygote *Pax3* mutant embryos (D–F) there is characteristic spinal bifidia and exencephaly. The dermomyotome of the somites is severely reduced (D). The forelimb (E) has no labelled (Pax3-positive) cells (black arrow). At the hindlimb level (F), there is also no sign of migrating cells (blue arrow).

Fredericks et al. 1995), is introduced into the *Pax3* gene there is complementation of the *Pax3* mutation. Indeed there is excessive production of hypaxial muscle progenitor cells. From this result we conclude that Pax3 functions through transcriptional activation *in vivo*, and we have isolated candidate co-activators.

Another homeo-domain containing transcription factor, Lbx1, is also implicated in the migration of cells from the somite. In *Lbx1* mutant embryos, muscle progenitor cells delaminate from the dermomyotome, but remain in the vicinity of the somite where they may adopt other cell fates (Schäfer & Braun, 1999). Interestingly, in the forelimb, dorsal muscle masses are especially affected, while ventral precursors appear to migrate correctly. It is not clear how the c-met/HGF system relates to this cell autonomous effect of Lbx1. Targets of Lbx1, which presumably acts as a transcription factor, have not yet been identified in muscle progenitor cells.

Another important question concerns the regulation of the *Pax3* and *Lbx1* genes. It has been suggested that *Lbx1* lies downstream of *Pax3* (Mennerich et al. 1998), but our data would suggest that it can be activated independently. Genes such as *Myf5* and *MyoD*, which regulate the myogenic cascade, are activated in the context of the somite by signalling molecules from the surrounding tissues (Tajbakhsh et al. 1998). These signals that specify the potential muscle fate of the multipotent cells of the somite include Wnts, and Wnt6, produced by the surface ectoderm, has been shown to be a candidate molecule for the activation of *Pax3* (Fan et al. 1997). *Pax3* transcripts are already detectable in presomitic mesoderm and the later effects of Pax3 on muscle progenitor cells in the somite may reflect the presence of stage- and site-specific transcriptional co-activators.

Expression of myogenic factors, proliferation and differentiation in the limb

Cells that migrate from the somite have not yet activated the myogenic determination genes and it is only when they reach the limb that they begin to express *MyoD* and *Myf5* (see Tajbakhsh & Buckingham, 1994). In the absence of these two factors, as in the case of the somite, cells which would normally form muscle adopt other cell fates (Kablar et al. 1999). In the presence of Myf5 alone the onset of myogenesis is delayed, suggesting that the level of Myf5 is insufficient initially (Kablar et al. 1997). This is in contrast to the somite, where *Myf5* is activated first and myogenesis proceeds in the absence of MyoD. Activation of *Myf5* and *MyoD* genes in the limb, as in the somite, may well depend on signalling molecules, such as the Wnts (Wnt7a) and Sonic hedgehog produced by the dorsal surface ectoderm and zone of polarizing activity, respectively. However, the regulatory sequences necessary for the expression of *Myf5* in the limb (see Hadchouel et al. 2000) are distinct from those which respond to Sonic hedgehog (Gustafsson et al. 2002) and drive *Myf5* expression in the epaxial dermomyotome (Teboul et al. 2002).

We also conclude that they are probably different from sequences responsible for other sites of *Myf5* transcription in the somite. The homeo-domain factor, Mox2, is present in muscle progenitor cells in the limb. In its absence, Myf5 transcripts are down-regulated, suggesting that Mox2 may act upstream of this myogenic factor gene (Mankoo et al. 1999). In this mutant, *Pax3* transcription is also reduced in the limb. MyoD is present. The transcription of *MyoD* in the limb may depend on the Six homeo-proteins, Six1 and Six4, acting with co-factors Eya and Dach (see Relaix & Buckingham, 1999).

Before skeletal muscle forms (Fig. 3), the muscle precursor cells, probably both before and after activation of *Myf5* and *MyoD*, undergo extensive proliferation in the limb. In chick embryos, MyoD was not detected in dividing myoblasts (Delfini et al. 2000), but in mouse both *Myf5* and *MyoD* are expressed in proliferating muscle cells and indeed they probably play a role in cell cycle regulation. Pax3, perhaps directly, and probably also through activation of *c-met,* may be involved in maintaining this proliferative phase. Some of the homeo-box factors already mentioned may also be important at this stage.

Another homeo-box factor, Msx1, is present in migrating muscle progenitor cells at the forelimb level and has been shown to keep cultured myoblasts dividing (see Houzelstein et al. 1999) and indeed its overexpression in differentiated muscle cells causes them to revert to a proliferative state (Odelberg et al. 2000). The FGF family has been implicated in myogenesis in the limb and it has been supposed that signalling through FGF receptors promotes myoblast proliferation (e.g. Edom-Vovard et al. 2001) and even migration to the limb bud (Webb et al. 1997), but recent results also point to a role at the level of muscle cell

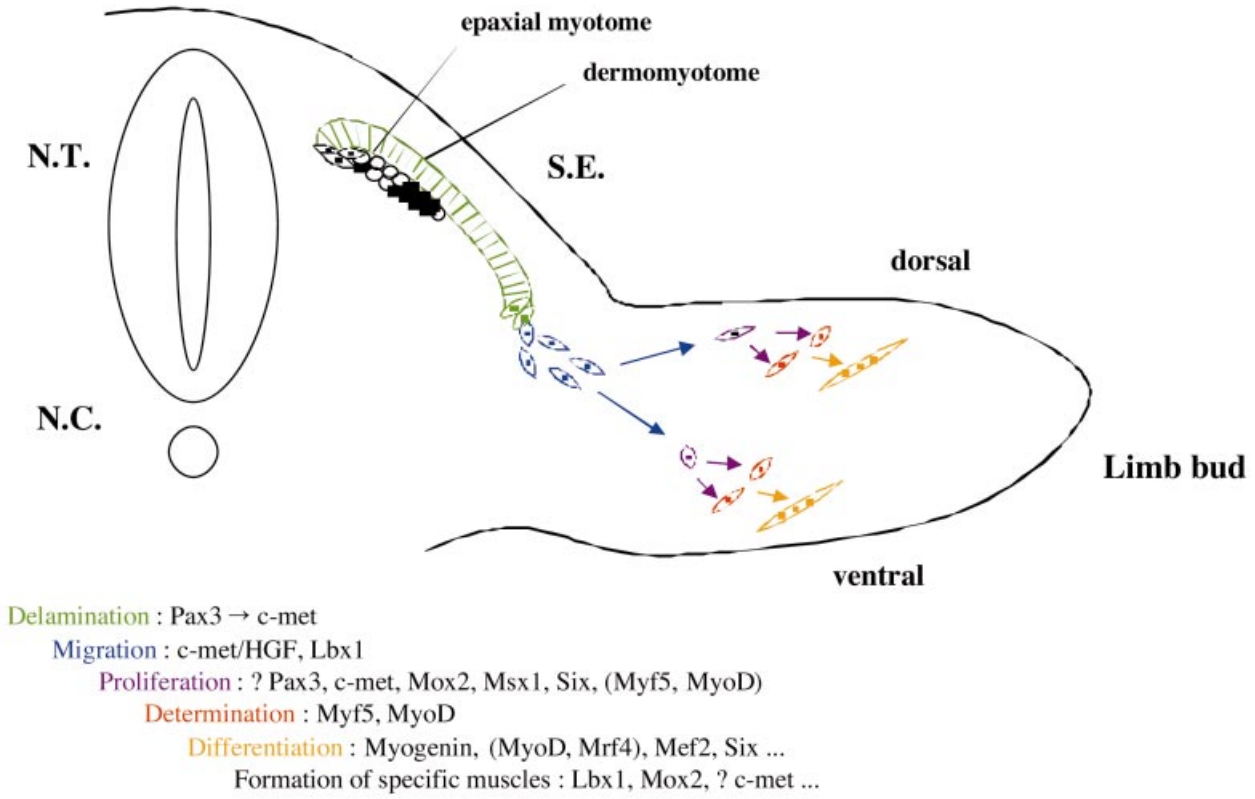

Fig. 3 Schematic representation of skeletal muscle formation in the limb, with the different stages and genes potentially involved at each stage. NC, notochord; NT, neural tube; SE, surface ectoderm.

differentiation. FGFR4, probably with FGF8 as its ligand, is required for the arrest of myoblast proliferation which is followed by the expression of muscle genes (Marics et al. 2002).

The activation of the differentiation programme depends on the presence of Myogenin and other differentiation factors such as Mef2. Muscle cell differentiation in the chick limb has been shown to be affected by Notch signalling, which, when stimulated by the over-expression of its ligand Delta, prevents MyoD expression in post-mitotic muscle cells (Delfini et al. 2000).

Genetic differences between limb muscles

As development proceeds, the dorsal and ventral muscle masses of the limb split into subdomains which will form the distinct muscles of the adult. As in the case of the location of the migrating muscle progenitor cells, this is thought to be programmed in part at least by the limb environment. These muscles will contain different proportions of slow and fast fibres, but otherwise they do not show notable differences in gene expression. A number of recent observations suggest, however, that they are intrinsically different in terms of the molecular regulation which led to their formation. In the *Lbx1* mutant mouse, specific limb muscles are missing or reduced (e.g. Schäfer & Braun, 1999; Gross et al. 2000). This effect reflects the failure of muscle progenitor cells leaving the somite to migrate correctly into the limb. Mutations of *c-met*, which partially compromise the function of this receptor by specifically uncoupling different intracellular signalling pathways, also show effects on particular muscles. The precursor cells appear to delaminate and migrate from the somite correctly but later proliferation (during secondary myogenesis) is affected (Maina et al. 2001).

The *Mox2* mutant mouse again shows effects on specific muscles, which are distinct from those seen with the *Lbx1* and *c-met* mutants. There does not appear to be a problem with migration; proliferation may be affected through a reduction in *Pax3* transcription, as well as the effect on *Myf5* transcription (Mankoo et al. 1999). This effect on *Myf5* may result in differences between limb muscles because of the presence of more than one regulatory sequence targeting *Myf5* expression

to the limb, as suggested by our results. Different sites of *Myf5* transcription in the limb may therefore be subject to different molecular regulators, one of which may be Mox2. However, it remains unclear why an effect on Myf5 in some limb muscles is not compensated for by MyoD, as in the case of the *Myf5* mutant mouse (Kablar et al. 1997).

It is striking in the *Mox2* and *Lbx1* mutants that the musculature of fore- and hindlimbs is not affected to the same extent. In the absence of Lbx1, for example, the hindlimb muscles are very severely affected while, in the forelimbs, cells migrate to the ventral muscle mass and flexor, but not extensor, muscles form (e.g. Schäfer & Braun, 1999; Gross et al. 2000). In contrast, in the *Mox2* mutant it is in the forelimbs that certain muscles are totally missing while effects on the hindlimb muscles are limited to a reduction in size (Mankoo et al. 1999).

Mox2 and Lbx1, like Pax3 and c-met, appear to be expressed equally in fore- and hindlimb muscle precursors. The phenotypes of *Mox2* and *Lbx1* mutants may reflect the presence of other factors which may affect the behaviour of muscle cells and which differ between fore- and hindlimbs, such as members of the Tbx family of T-box transcription factors; Tbx5, for example, is expressed in the mesenchymal cells of the forelimbs and Tbx4 in the hindlimbs (Gibson-Brown et al. 1996).

Since on the anterior/posterior developmental gradient, which also governs somitogenesis, the forelimbs form before the hindlimbs there may also be a temporal parameter in the impact of the mutation. Within a single limb, the timing of muscle cell migration from the somite may be important. In the chick it has been shown that cells which migrate first tend to contribute to muscles rich in slow fibre types while cells which migrate later form fast muscles (Van Swearingen & Lance-Jones, 1995). In the mammalian case it does not appear to be a question of slow vs. fast, but it could well be that the progenitor cells for some muscle masses migrate first and that the regulatory environment changes with time.

Distal muscles, such as those of the hand, may be formed by an earlier migratory population than more proximal muscles. More evidently, there is an anterior/posterior gradient corresponding to the body axis, particularly noticeable during the maturation of muscles in the fetal limb (Ontell et al. 1993). The mouse mutants discussed do not appear to reflect proximal/distal or anterior/posterior phenomena. A possible link with temporal differences in the contribution of muscle cell progenitors to different muscle masses should be possible to test with conditional mutations.

Muscle growth and regeneration

The origin of embryonic skeletal muscle is well established in its broad outlines, whereas it is less clear what happens later in the perinatal period. The first muscle fibres that appear are known as primary fibres (about embryonic day (E) 11–14 in the mouse limbs), around which secondary fibres form at the time when innervation begins to be established (about E14–16) (see Ontell & Kozeka, 1984). Primary and secondary fibres can be distinguished morphologically and show some differences in muscle gene expression. It has been proposed that some myoblasts remain quiescent in the embryonic limb due to the presence of TGFβ receptors; this signalling pathway blocks differentiation (Cusella-De Angelis et al. 1994). Later, other signals stimulate a wave of proliferation giving rise to a population of so-called secondary myoblasts which will differentiate to form secondary fibres (Ross et al. 1987). Secondary fibres acquire the characteristics of fast fibres, whereas primary fibres tend to become slow fibres. Subsequently, the muscle masses undergo very extensive growth in the fetal period and postnatally.

Later muscle cell progenitors

The precursor cells of adult muscle are known as satellite cells because they lie along the muscle fibre under the basal lamina (Mauro, 1961). Embryonic and fetal myoblasts and adult muscle satellite cells can be distinguished by their behaviour on differentiation in cell culture (Cossu & Molinaro, 1987), in terms of morphological criteria, drug resistance and gene expression. More recently, it has been suggested that fetal myoblasts, unlike embryonic myoblasts, may not be derived from somites, but may have an endothelial origin. They express some endothelial cell markers and it has been shown that cells of the embryonic dorsal aorta can contribute to fetal muscle (De Angelis et al. 1999). The difficulty is to estimate the extent of this mesangioblast contribution, whether it represents a major source of fetal muscle progenitors or not. It is attractive to think that the blood vessels which invade all the musculature may contain cells which contribute to muscle growth.

In collaboration with J-F. Nicolas, who developed this genetic approach, and S. Eloy-Trinquet, we have begun

a retrospective clonal analysis to look at cellular behaviour in the muscles of mice in the perinatal period. These experiments depend on the detection of β-galactosidase in clones which have undergone a rare recombination event converting a laacZ transgene carrying a duplication into a functional lacZ sequence. The transgene is targeted into an *actin* gene expressed in muscle. Embryonic muscle clones in the somite have been analysed using this approach (Eloy-Trinquet & Nicolas, 2002). In perinatal muscle, labelled cells are grouped together such that in the most striking cases a single muscle is almost entirely β-galactosidase positive. This suggests an oligoclonal origin. One possible scenario is that somitically derived 'founder' cells serve as a scaffold on which each muscle grows by the addition of other cells which tend to have a common clonal origin.

When muscle is injured, satellite cells become activated to divide and then differentiate to form a new fibre. The origin of satellite cells is unclear. They also express some endothelial cell markers (Beauchamp et al. 2000). Recently, there has been considerable interest in the presence of adult stem cells, which by definition are multipotent cells, with considerable proliferative potential. Satellite cells only have a limited capacity for self-renewal, which means that under pathological conditions skeletal muscle degenerates. Stem cells may prove to be of therapeutic value for cell therapy if it becomes feasible to mobilize them appropriately. It has been shown that a stem cell fraction in bone marrow can provide skeletal muscle progenitors (Ferrari et al. 1998), although the efficiency of this process is very low. Adult skeletal muscle, of the limb for example, also contains a so-called stem cell population which can be separated on similar criteria to those applied to bone marrow stem cells (Gussoni et al. 1999) with which they have markers in common. These cells also appear to be able to contribute to muscle and blood. It is not clear whether they give rise to satellite cells or integrate muscle fibres through another route. Again this is a rare event. The origin of the so-called muscle stem cells is unknown; perhaps they arise from blood vessels/blood cells or from connective tissue (Young et al. 1995) within the muscle.

Regulatory genes of adult muscle

The myogenic regulatory factors are also present during the formation of adult muscle fibres. *Myf5* (*Myf5-nlacZ*) is transcribed in most satellite cells (Beauchamp et al. 2000), which accumulate MyoD as they differentiate. In the absence of MyoD, muscle growth and regeneration are affected (Megeney et al. 1996). It is Pax7, the orthologue of Pax3, which has been shown to play a crucial role in the formation of adult skeletal muscle (Seale et al. 2000). This *Pax* gene is expressed in satellite cells and in *Pax7* mutant mice satellite cells are absent.

These observations were made on limb muscle, on the gastrocnemius. With a mouse line, in which an nlacZ reporter has been targeted to an allele of *Pax3,* we have detected expression of this *Pax* gene in adult muscle also (Fig. 4). Not all satellite cells are absent from the *Pax7* mutant mouse, suggesting that Pax3 can play a similar role in conferring muscle precursor cell identity. Interestingly, the presence of Pax3 in satellite cells depends on the muscle. This is not a question of fibre type, but is muscle mass specific. For example, in the limb, the satellite cells of most muscles are Pax7 positive, Pax3 negative, but the satellite cells of the gracilis muscle are Pax3 positive. In the early embryo, all developing muscle masses are derived from Pax3-positive cells. However, Pax3 expression is only maintained in the muscles which will remain positive in the adult. As in the case of *Lbx1* or *Mox2* mutants, this points to differences in the regulatory strategies underlying the formation of muscles, which differ by their position in the limb or the body. We do not know yet what determines these differences, but the fact that muscle diseases in which a mutation in a gene, which is expressed in all skeletal muscles, only affects some muscles may not be unrelated to this phenomenon.

Skeletal muscle cells in the limb may not be as naive as was first thought. There may be intrinsic differences between them from the start, or they may acquire differences, as a result of their position, which then become part of their own identity.

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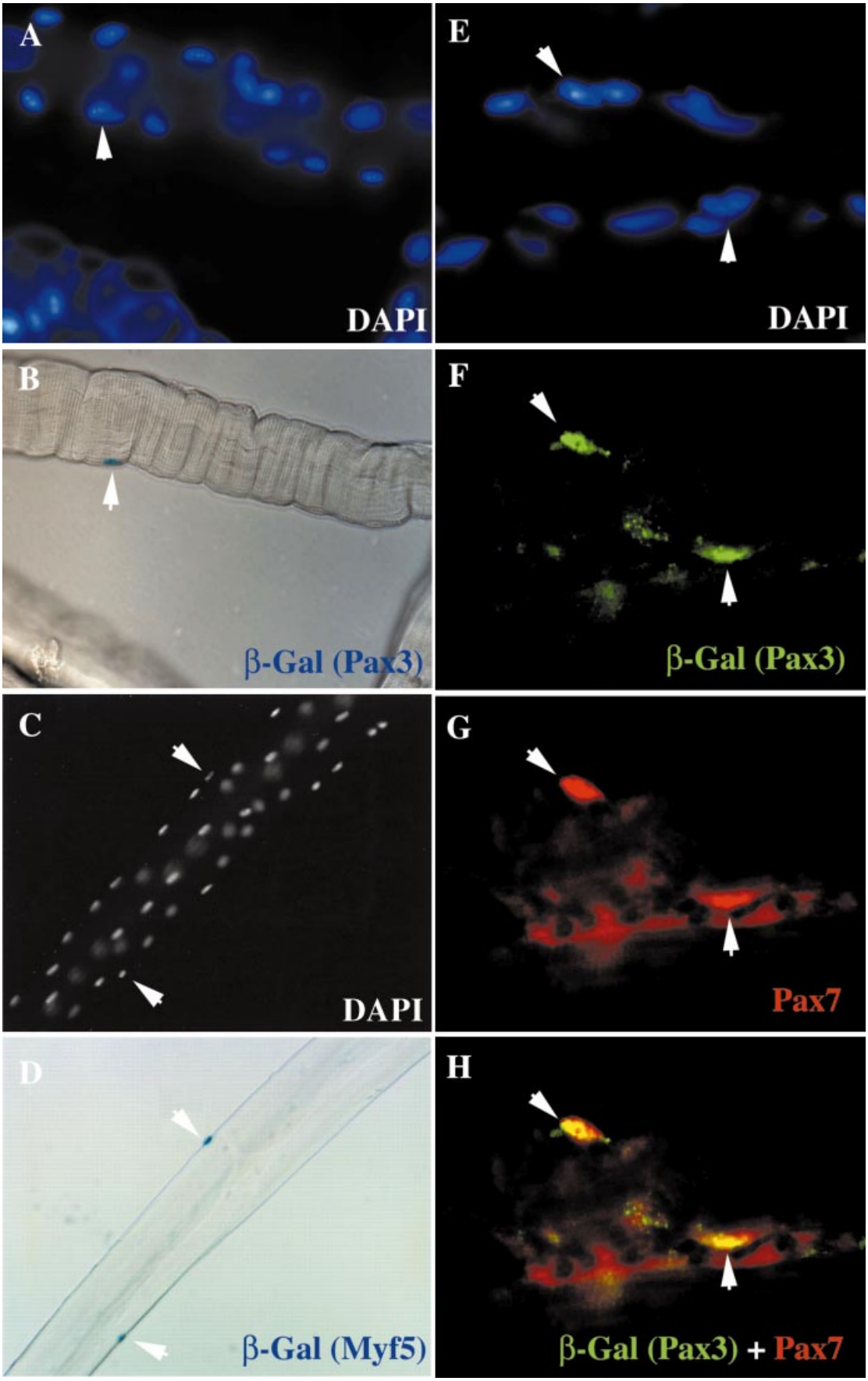

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Fig. 4 Myogenic genes expressed in adult muscle satellite cells. (A) Dapi staining of nuclei in an isolated adult muscle fibre. (B) The same fibre, showing a Pax3-positive satellite cell (from an nlacZ-targeted allele). (C) Dapi staining of nuclei in an isolated adult muscle fibre. (D) The same fibre showing two Myf5-positive satellite cells (from an nlacZ-targeted *Myf5* allele). (E) Dapi staining of nuclei in an isolated adult muscle fibre. (F) The same fibre showing Pax3-positive (β-Gal) satellite cells. (G) The same fibre showing that the same cells (see H) are expressing Pax7. (H) A merge of F and G. C and D are modified from Beauchamp et al. (2000).

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